EXHIBIT C

Combinatorial Cassette Mutagenesis as a Probe of the Informational Content of Protein Sequences

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A method of combinatorial cassette mutagenesis was designed to readily determine the informational content of individual residues in protein sequences. The technique consists of simultaneously randomizing two or three positions by oligonucleotide cassette mutagenesis, selecting for functional protein, and then sequencing to determine the spectrum of allowable substitutions at each position. Repeated application of this method to the dimer interface of the DNA-binding domain of λ repressor reveals that the number and type of substitutions allowed at each position are extremely variable. At some positions only one or two residues are functionally acceptable; at other positions a wide range of residues and residue types are tolerated. The number of substitutions allowed at each position roughly correlates with the solvent accessibility of the wild-type side chain.

It has been more than 20 years since Anfinsen and his colleagues showed that the sequence of a protein contains all of the information necessary to specify the three-dimensional structure (*1*). However, the general problem of predicting protein structure from sequence remains unsolved. Part of the difficulty may stem from the complexity of protein structures. Although some 200 protein structures are known, no rules have emerged that allow structure to be related to sequence in any simple fashion (*2*). The problem is further complicated by the nonuniformity of the structural information encoded in protein sequences. Some residue positions are important, and changes at these positions can tip the balance between folding and unfolding (*3–7*). Other residues are relatively unimportant in a structural sense and a wide range of substitutions or modifications can be tolerated at these positions (*3, 7–9*).

If only a fraction of the residues in a protein sequence contribute significantly to the stability of the folded structure, then it becomes important to be able to identify these residues. We now describe the results of genetic studies that allow the importance of individual residues in protein sequences to be rapidly determined. Specifically, we determine the spectrum of functionally acceptable substitutions at residue positions near the dimer interface of the NH_2-terminal domain of phage lambda (λ) repressor (*10*). The NH_2-terminal domain binds to operator DNA as a dimer, with dimerization mediated by hydrophobic packing of α helix 5 of one monomer against α helix 5′ of the other monomer (*11*) (Fig. 1, A and B). Without helix 5 there are no contacts between the subunits (Fig. 1C). By applying combinatorial cassette mutagenesis to the helix 5 region, we find that the number and spectrum of allowable substitutions within helix 5 are extremely variable from residue to residue. In most cases, this variability can be rationalized in terms of the fractional solvent accessibility of the wild-type side chain.

General strategy. For our studies, we used a plasmid-borne gene that encodes a functional, operator-binding fragment (residues 1–102) of λ repressor (*12*). The binding of the 1–102 fragment to operator DNA depends on dimerization which, in turn, depends on the helix 5–helix 5′ packing interactions (*11, 13*). Thus, if a 1–102 protein retains normal operator-binding properties, we can infer that it is able to dimerize normally.

Mutagenesis of the helix 5 region was performed by a combinatorial cassette procedure. One example of this method, in which codons 85 and 88 are mutagenized, is illustrated in Fig. 2. On the top strand, the mutagenized codons are synthesized with equal mixtures of all four bases in the first two codon positions and an equal mixture of G and C in the third position. The resulting population of base combinations will include codons for each of the 20 naturally occurring amino acids at each of the mutagenized residue positions. On the bottom strand, inosine is inserted at each randomized position because it is able to pair with each of the four conventional bases (*14*). The two strands are then annealed and the mutagenic cassette is ligated into a purified plasmid backbone.

To identify plasmids encoding functional protein, we selected transformants for plasmid-encoded resistance to ampicillin and for resistance to killing by *cI*⁻ derivatives of phage λ. The latter selection requires that the cell express 1–102 protein that is active in operator binding (*15*). For each mutagenesis experiment, many independent transformants were chosen, single-stranded plasmid DNA was purified, and the relevant region of the 1–102 gene was sequenced. The resulting set of sequences provides a list of functionally acceptable helix 5 residues.

Substitutions in the helix 5 region. In separate experiments with different mutagenic cassettes, the codons for helix 5 residues 85 and 88; 86 and 89; 90 and 91; 84, 87, and 88; and 84, 87, and 91 were mutagenized, and genes encoding active 1–102 proteins were selected. In some cases, the survival frequency was low. For example, only 17 of 60,000 transformants passed the selection after randomization of codons 84, 87, and 88. In this case, each active candidate was sequenced. By contrast, 1,200 of 50,000 transformants passed the selection in the mutagenesis of positions 86 and 89 (*16*). In this case, we picked 50 candidates for sequence analysis. Overall, 150 active genes were sequenced (Table 1). In addition, we sequenced

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approximately 40 genes that had been mu[illegible]nized, but not subjected to a functional selection. These [illegible] as controls for the efficiency of mutagenesis and also provide examples of helix 5 mutations that result in inactive 1–102 proteins (Table 1).

Many of the active sequences contain at least two residue changes compared to wild type. In principle, some of these changes could be compensatory; for example, residue X might be functionally allowed at position 85 only in combination with residue Z at position 88. This cannot be generally true, however, because most residue changes at one position were recovered in combination with several different changes at the other position or positions. It is therefore likely that most substitutions that are functionally acceptable in multiply mutant backgrounds would also be allowed as single substitutions. In Fig. 3, we show the spectrum of functionally acceptable substitutions at residue positions 84 to 91.

From the list of allowed substitutions, several conclusions may be drawn concerning the [illegible]tural requirements at various positions in helix 5. We n[illegible] consider these residue positions in order of decreasing "informational content," where this term is roughly defined as a value that decreases as the number of allowed substitutions increases. Thus, the informational content of a residue position is highest if only the wild-type amino acid is allowed and is lowest if each of the 20 naturally occurring amino acids is allowed.

Table 1. Sequences for the helix 5 region of active and inactive mutants obtained by combinatorial cassette mutagenesis. Active mutants are resistant to phage λKH54; these are grouped by cassette, with the wild-type sequence at the top of each group and randomized positions in boldface. Asterisks indicate sequences of mutants obtained in the absence of a functional selection. The activity of these mutants was subsequently determined by a screen. Numbers next to sequences indicate the number of times particular mutant sequences were obtained. Numbers at the tops of the columns indicate amino acid positions. The one-letter abbreviations for the amino acids are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Active

85 90		85 90		85 90		85 90	
IYEMYEAV		-L--V---		--Y--S--		--E--L--*	
I--MF---	2	-L--M---		--Y--T--		--E--E--	
I--MY---	4	-I--F---		--W--A--		--E--G--*	
I-AMA---		-I--A---		--W--C--		--G--E--	
I-DMY---		-I--S---		--W--Q--			
I--MA---	3	-Q--Y---		--W--S--	3	IYEMYEAV	
I--MI---		-S--Y---		--W--K--*		------WA	4
I--LF---		-S--W---		--W--D--		------WL*	
I--LW---		-T--Y---		--W--D--*		------WS	
		-T--W---		--W--E--		------HV	
IYEMYEAV		-T--A---		--W--G--	3	------AV	6
I--M---V		-R--F---		--A--E--	3	------AC	2
I--M---T		-R--W---	4	-SM--A--		------AL	4
I--L---T		-R--A---	3	--M--S--	3	------AI	
		-R--C---		--L--S--	2	------AT	2
IYEMYEAV		-E--F---		--L--T--		------VA	
-Y--F---		-G--Y---		--L--E--		------VC	2
-W--W---	2			--L--E--*		------MA	
-W--A---		IYEMYEAV		--I--S--		------LA*	
-A--Y---		--F--M--		--Q--S--		------QV	
-V--Y---	2	--F--L--		--S--Q--		------QT	
-V--A---	3	--F--I--		--S--E--		------SV	3
-C--F---	2	--F--Q--		--T--E--		------SC	2
-C--A---		--F--S--	3	--D--L--		------SL	5
-L--F---		--F--T--		--D--Q--		------ST	
-L--W---		--F--R--		--D--D--*		------GC	
-L--A---		--F--E--		--E--L--	2	------GI	
						------GT	

Inactive

85 90		85 90		85 90		85 90	
A--VA---*		P--DS---*		Q--CS---*		------RN*	
P--PL---*		R--TR---*		A--TP---*		------EA*	
P--TN---*		T--TV---*		S--TK---*		------KV*	
R--NP---*		R--VI---*		T--LN---*		------VM*	
P--LL---*		L--PL---*		A--SL---*		------PA*	
A--IL---*		I--LL---*		R--WS---*		------NQ*	
T--KP---*		K-AIV---*		------PR*		------ME*	
Q--RV---*		C--YT---*		------PP*	5	------AY*	
H-DVR---*							

Positions 84 and 87 in particular stand out as having a high informational content. Ile appears to be the only acceptable residue at position 84. Both Met and Leu are residues of similar size and hydrophobicity, and are the only two residues that appear to be functional at position 87. The side chains of Ile^{84} and Met^{87} form a major part of the helix-helix packing interaction at the dimer interface, where Ile^{84} of one subunit packs against Met$^{87'}$ of the other subunit, and vice versa (Fig. 4). This cluster of four residues also contacts the globular portions of the domain. Solvent accessibility calculations by the method of Lee and Richards (*17*) show that the Ile^{84} and Met^{87} side chains are almost completely buried (92 to 98 percent solvent inaccessible) in the structure of the dimer. We assume that replacement of Ile^{84} or Met^{87} with smaller side chains would diminish dimerization because hydrophobic and van der Waals interactions would be lost. In fact, mutant repressors containing Ser^{84} or Thr^{87} are defective in dimerization (*13, 18*). Replacing Ile^{84} or Met^{87} with larger residues would also be expected to be detrimental because substantial structural rearrangements would be required to accommodate larger side chains.

Seven residues (Leu, Ile, Val, Thr, Cys, Ser, and Ala) are functionally acceptable at position 91. Aromatic residues, charged residues, and strongly hydrophilic residues are not found. The wild-type Val side chain is partially buried in the dimer structure, with the Cγ2 methyl group packing against the Cδ1 methyl group of the Ile$^{84'}$ side chain. Although some of the acceptable substitutions such as Ile and Thr could make equivalent packing contacts, others such as Ala and Ser could not.

Nine residues (Trp, His, Met, Gln, Leu, Val, Ser, Gly, and Ala) are acceptable at position 90. There is a surprisingly large range in both the acceptable size and hydrophilicity of these side chains. This is especially true as the Cβ methyl group of the wild-type Ala is almost completely buried in the structure of the dimer and, at first glance, it would appear that larger side chains could not be accommodated. However, the inaccessibility of the Cβ methyl group of Ala^{90} is largely caused by the Lys$^{67'}$ side chain, which packs against it. By rotating the Lys$^{67'}$ side chain away, we were able to introduce a Trp^{90} side chain by model-building without steric clashes. Rotation of the Lys$^{67'}$ side chain away from Ala^{90} should not be energetically costly and, in fact, is observed in crystals of the NH$_2$-terminal domain bound to operator DNA (*19*).

Nine different residues (Trp, Tyr, Phe, Met, Ile, Val, Cys, Ser, and Ala) are functionally acceptable at position 88. There are large variations in the sizes and volumes of the acceptable side chains, although most are relatively hydrophobic. Charged residues and other strongly hydrophilic residues are not observed. In the wild-type dimer (*11*), the aromatic ring of Tyr^{88} stacks against the ring of Tyr$^{88'}$. The side chains of Trp, Phe, Met, Ile, and Val could probably form some type of packing interaction at this position, although those of Ala and Ser could not. It is known that the presence of Cys at position 88 allows a stable Cys^{88}–Cys$^{88'}$ disulfide bond, which links the monomers in a conformation that is active in operator binding (*20*).

Positions 85, 86, and 89 show considerable variability. At each of these positions, 13 different amino acids were found to function. At positions 85 and 86, aromatic, hydrophobic, polar, and charged residues are all acceptable. At position 89, aromatic residues were not represented, but each of the remaining classes was observed. In

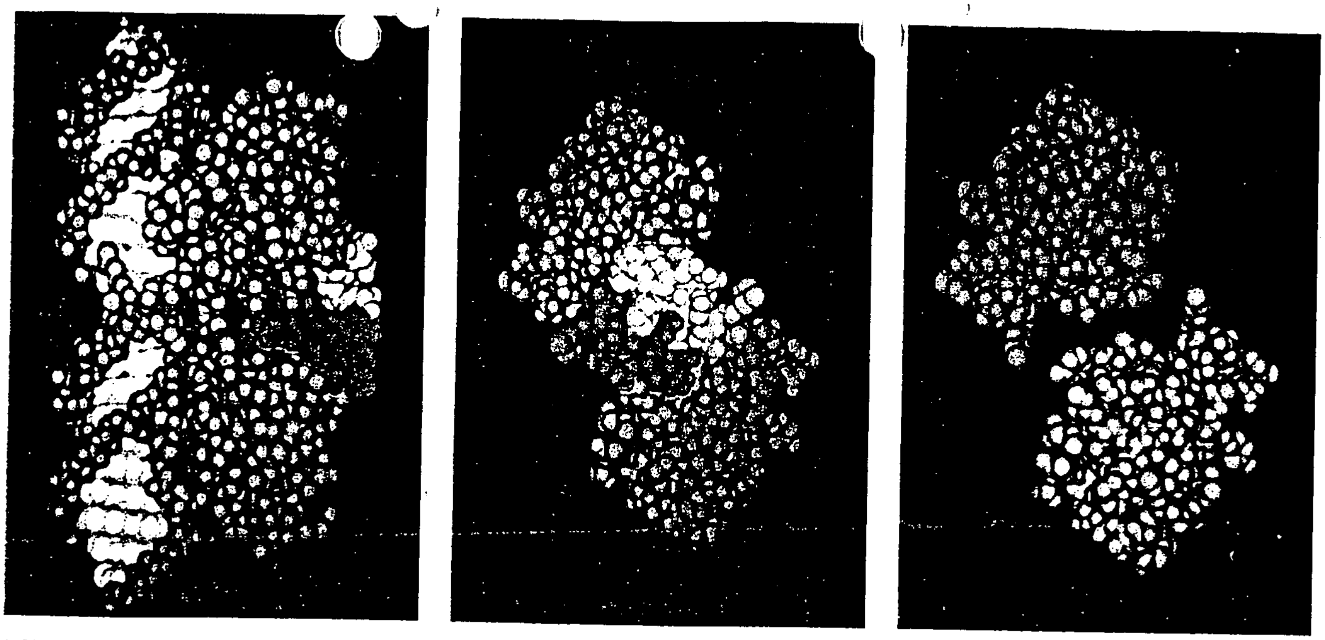


Fig. 1. Three views of the DNA-binding domain of λ repressor, showing the role of helix 5 in dimerization. (**A**) Proposed complex of repressor dimer with operator DNA (*11*). Helix 5 of each monomer is colored more lightly than the globular portion of that monomer. (**B**) Free repressor dimer, rotated 90° from the view in (A), to show the "back side" of the molecule. (**C**) Dimer with helix 5 of each monomer removed. This view illustrates the role helix 5 plays in mediating dimerization (*26*).

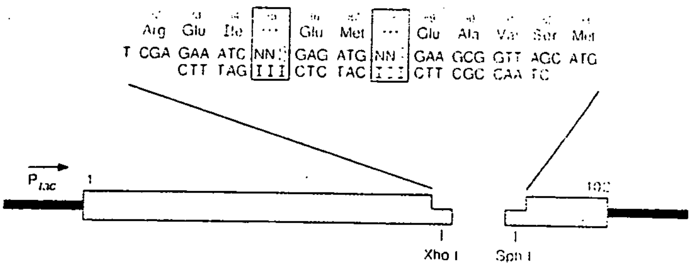

Arg Glu Ile ··· Glu Met ··· Glu Ala Val Ser Met
T CGA GAA ATC NN: GAG ATG NN: GAA GCG GTT AGC ATG
CTT TAG III CTC TAC III CTT CGC CAA TC
P_{tac}
1
102
Xho I
Sph I

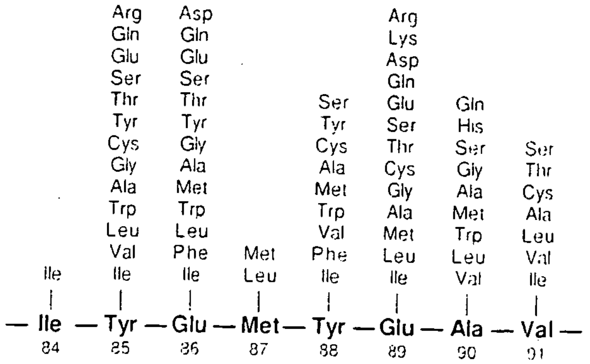
Fig. 2. Schematic diagram showing the combinatorial cassette mutagenesis procedure. At positions indicated as N, an equal mixture of A, G, C, and T was used during oligonucleotide synthesis. At positions indicated as I, inosine was used. After synthesis, the oligonucleotides were phosphorylated, annealed, and ligated into the Xho I–Sph I backbone of plasmid pJO103. Plasmid pJO103 is an M13 origin plasmid with the 1–102 gene under control of a *tac* promoter; the region of the 1–102 gene encoding residues 82–93 (the small Xho I–Sph I fragment) is replaced by an unrelated 1.9-kb Xho I–Sph I "stuffer" fragment. Ligated DNA was transformed into *Escherichia coli* strain X90 F'$lacI^Q$ cells (*27*), and ampicillin-resistant colonies were selected in the presence or absence of phage λKH54. Candidates that survived the selection were cross-streaked against a series of virulent derivatives of phage λ to confirm their immunity properties [strains and methods are described in (*21*)]. Single-stranded plasmid DNA was purified from an M13RV1 transducing lysate as described (*28*), and DNA sequences were determined by the dideoxy method (*29*).

the wild-type dimer, the side chains of Tyr[85], Glu[86], and Glu[89] are relatively solvent accessible.

Several amino acids are significantly underrepresented among the active sequences. For example, Pro is never found. This cannot be an artifact of our mutagenesis procedure because Pro is frequently observed among the unselected mutant sequences (Table 1). We conclude that Pro is not found among the functional sequences because it is selected against; its presence would presumably disrupt the α-helical structure and thereby the helix-helix packing at the dimer interface.

His, Asn, and Lys are also underrepresented among the functional helix 5 sequences. These residues are presumably not acceptable at positions 84 and 87, where the informational content is extremely high, and may not be acceptable at positions 88 and 91, where the functional substitutions are generally hydrophobic in character. The acceptability of these residues at positions such as 85 and 86 is difficult to assess from our experiments because the codons for these residues are present at reasonably low frequencies even among the unselected sequences. In these cases, we probably have not sequenced a large enough number of candidates to be confident that all acceptable substitutions have been identified. In fact, data from reversion studies (*21*) and suppressed amber studies (*22*) show that His[85] and Lys[86] are acceptable substitutions in the context of the intact λ repressor molecule.

Informational content and protein structure. We have combined an efficient combinatorial mutagenesis procedure and a functional selection to probe the informational content of the eight residues that form the major part of the dimerization interface of the NH_2-terminal, operator-binding domain of λ repressor. At two of these eight residue positions, the functionally acceptable choices are highly restricted. For example, we analyzed 17 functional genes in which codon 84 had been randomized and recovered the wild-type residue, Ile, in every case. This is clearly a position of high



— Ile 84	— Tyr 85	— Glu 86	— Met 87	— Tyr 88	— Glu 89	— Ala 90	— Val 91 —
Ile	Arg Gln Glu Ser Thr Tyr Cys Gly Ala Trp Leu Val Ile	Asp Gln Glu Ser Thr Tyr Gly Ala Met Trp Leu Phe Ile	Met Leu	Ser Tyr Cys Ala Met Trp Val Phe Ile	Arg Lys Asp Gln Glu Ser Thr Cys Gly Ala Met Leu Ile	Gln His Ser Gly Ala Met Trp Leu Val	Ser Thr Cys Ala Leu Val Ile

Fig. 3. Functionally acceptable residues in the helix 5 region. The amino acids are listed from top to bottom in order of increasing hydrophobicity according to the scale of Eisenberg *et al.* (*30*).

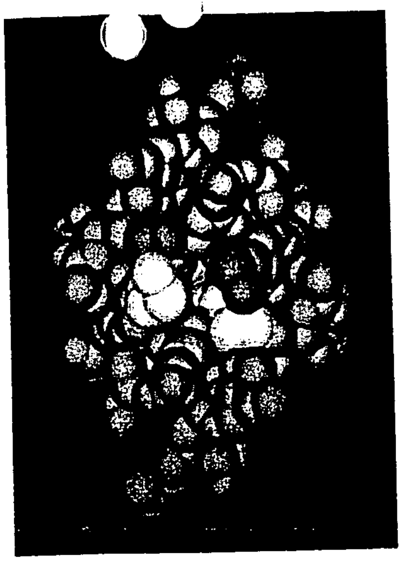


Fig. 4. Helix 5 residues high in informational content. The two isolated helix 5 regions of the protein are shown in green and blue. Ile[84] and Met[87] from the green helix are shown in yellow; Ile[84] and Met[87] from the blue helix are shown in red.

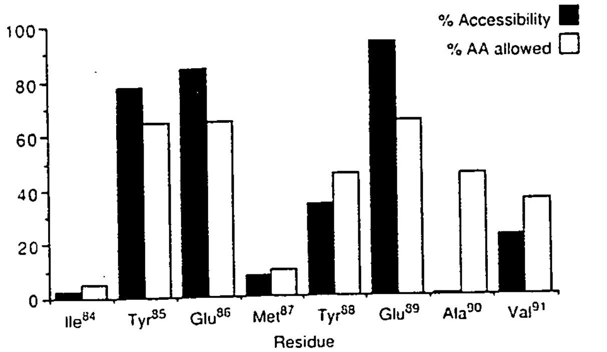


Fig. 5. Correlation between the solvent accessibility and the number of functionally acceptable substitutions. Hatched bars indicate the percentage of the 20 naturally occurring amino acids that are functionally acceptable at a residue position. Black bars indicate the fractional solvent accessibility of the wild-type side chain in the dimer. Solvent accessibilities for the NH_2-terminal domain dimer (*11*) were computed using a 1.4 Å probe by the method of Lee and Richards (*17*). Fractional accessibilities were obtained by dividing by the appropriate side chain accessibilities calculated for the monomer. The fractional accessibilities change only slightly if the side chain accessibilities in the reference tripeptide Ala-X-Ala (*17*) are used instead as the reference state.

informational content. The informational content is also high at position 87, where Met and Leu are the only acceptable residues. By contrast, the remaining positions have moderate to low informational contents. For example, among 38 functional genes in which codon 85 had been randomized, the wild-type residue was recovered only once, and 12 other residues, differing in size and chemical properties, were recovered in the remaining cases. This is clearly a position of low informational content. It is striking that most of the structural determinants of dimerization in this eight-residue segment reside in two residues only. The remaining positions are surprisingly tolerant of a wide range of substitutions. If this high level of tolerance is generally true of protein sequences, then the problem of understanding and predicting structure may rest largely on the ability to identify those few residues that are crucial.

The positional variability of the informational content in helix 5 can, in general, be rationlized in terms of the solvent accessibility of the wild-type residues in the crystal structure (*11*). There is a rough correlation between the number of acceptable substitutions and the fractional extent to which the wild-type side chain is solvent accessible (Fig. 5). At exposed surface positions such as 85, 86, and 89, we find that many different residues and residue types can be functionally accommodated. By contrast, at positions such as 84 and 87, where the wild-type side chain is almost completely buried, we find that the functionally acceptable residue choices are extremely restricted. There is one apparent exception to the simple rule that buried residues are high in informational content. Ala[90] is inaccessible to solvent in the crystal structure, and yet we find that many substitutions are allowed at this position. However, the inaccessibility of the Ala[90] side chain to solvent is not due to close packing at the dimer interface, but rather to an interaction with a nearby surface side chain. This side chain can presumably move to allow larger side chains to be accommodated at position 90. Examples of this type demonstrate the need to distinguish between two types of buried side chains: those that can become exposed by relatively minor rearrangement of other side chains, and those that are tightly packed in the hydrophobic core.

There is no reason to assume that there should always be a strict correlation between the solvent accessibility of a residue and the structural informational content of that position. For one thing, the chemical properties of the 20 amino acids are not related in any simple linear fashion. Moreover, the structural importance of some residues in proteins almost certainly stems from interactions other than simple hydrophobic packing. Nevertheless, the closely packed nature of protein interiors (*23*) provides a simple molecular explanation for the structural importance of buried residues, and destabilizing mutations are commonly found to affect hydrophobic core residues (*3–7*). By contrast, missense mutations or chemical modifications that affect surface residues are often found to have little or no influence on protein stability (*3, 7, 8*). Thus, it is reasonable that solvent accessibility should be an extremely important determinant of the informational content of a residue position.

Our overall strategy for rapidly probing informational content should be broadly applicable to a wide range of protein structure-function problems in systems where genetic selections or screens can be devised. The method consists of three basic elements: (i) the use of cassette mutagenesis to introduce extremely high levels of targeted random mutagenesis; (ii) the use of a functional selection to identify genes encoding active proteins; and (iii) the use of rapid DNA sequencing methods to determine the spectrum of functionally acceptable residues in a relatively large number of candidates. Our method of combinatorial cassette mutagenesis (Fig. 2) allows several residue positions to be mutagenized at the same time and, in principle, generates a mutant population in which each of the 20 amino acids is represented at each mutagenized position (*24*). When two or three codons are mutagenized at the same time, the entire analysis is able to proceed more rapidly. Moreover, at this level of mutagenesis most two-residue and three-residue combinations should be present in the mutagenized population and should be recovered if they result in a functional protein. In our study of the packing of the 84 and 87 side chains, we recovered only two (Ile[84] with Met[87] and Ile[84] with Leu[87]) of the 400 possible residue combinations. Thus, because both positions were mutagenized in the same experiment, we are able to conclude that there are not significantly different ways of packing the dimer interface.

In principle, data like that shown in Fig. 3 could be generated for an entire protein sequence, and additional experiments could be devised to determine whether the positions of high informational content were important for structure or function. For proteins of unknown structure, such data might be quite useful for structural predictions. First, current predictive algorithms could be applied to the family of related sequences generated by our method, as each of these sequences is able to form the same basic structure. Second, because of their fundamental repeats, α-helical and β-strand regions might be recognized by characteristic patterns of high and low informational content. Third, the positions of highest structural informational content should include the residues involved in formation of the hydrophobic core of the protein. This information might prove useful in combination with [illegible] tertiary template ideas recently proposed (25).

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